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PARAMYXOVIRUSES OF THE MORBILLI GROUP IN THE WILD HEDGEHOG *ERINACEUS EUROPEUS*

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Summary.—Dead and sick hedgehogs (*Erinaceus europeus*) were examined, together with apparently healthy individuals, and paramyxo virus of the morbilli group was isolated. One animal's symptoms were similar to those caused by canine distemper and the virus isolate from faecal suspensions from this animal were antigenically related in various degrees to measles, canine distemper, rinderpest and PPRV viruses. Isolates from normal hedgehogs were found to belong to the same group. The variability and host specificity of members of paramyxo morbilli group virus are discussed and the role of natural infections of wild communities is considered in relation to disease in domestic animals and man.

OVER THE YEARS there have been occasional undocumented reports that describe the findings of hedgehogs (*Erinaceus europeus*) either dead in unusual numbers or in an unusual state of stress. Some reports mention animals with paralysis of the hind legs or with some lesser ambulatory malfunction; others refer to hedgehogs found in an emaciated condition which refuse to accept food and soon die.

As we were involved in a research programme associated with the occurrence of viruses in wild-animal populations, we were interested to hear of the finding of a number of dead hedgehogs of various ages in one small area of parkland in North Oxford. The behaviour of one animal in particular was observed to be unusual and to resemble that reported in a classical paper by Boys Smith (1976), who described the circling behaviour of a hedgehog in the garden of the Master's Lodge at St John's College, Cambridge. Unlike the Cambridge hedgehog, however, the Oxford individual ran round in clockwise circles, was blind, walked with an unusual gait, and had difficulty in breathing. Its footpads were swollen and badly ulcerated and there was a discharge from both nose and eyes.

This paper reports the isolation of a virus of the paramyxo group from faecal material taken from this animal on two occasions two months apart, and from its lungs, and also reports the subsequent isolation of other viruses of the same group from several apparently healthy hedgehogs. Some of the implications of these findings are discussed.

MATERIALS AND METHODS

Samples were taken from the diseased hedgehog (Hh1) and at various times through the year from 5 other normal animals.

Faecal samples were taken at the time of capture and in the case of Hh1 blood was taken by heart puncture under Nembutal anaesthesia. Faecal samples were also collected during the year from 5 other wild specimens. Samples were stored at $-70°$ as 10% or 20% suspensions in phosphate-buffered saline (PBS) with 50 μg/ml gentamicin and 25 μg/ml fungizone (Amphotericin B, Gibco Biocult). They were clarified by centrifugation at 3000 r.p.m. for 15 min and filtered through a 0·4μm Millipore filter. Some samples were partly purified and concentrated by polyethylene glycol precipitation for electron microscopy.

A second two-phase separation method using an ethoxy and butoxy ethanol mixture and 2·5M phosphate buffer, pH 7·5, was also used to

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isolate possible enteroviruses present (Kitano, Haruna and Watanabe, 1961).

Histology.—Organs were removed for histology and for possible virus isolation. The former were fixed in formol saline and paraffin sections were stained with haematoxylin and eosin (H. & E.) or iron haematoxylin (IH). Specimens for virus isolation were stored at −20° in PBS and were used at a later date to make smears to be tested by immunofluorescent staining techniques.

Tissue culture.—All inoculations were made in cultures of the continuous cell lines of L929 (mouse fibroblast), BHK C13-21 (baby hamster kidney fibroblasts) and Vero (monkey kidney) cells. L929 cells were maintained in Modified Eagle's Medium with Earle's salts (Gibco Biocult Ltd), BHK C13-21 in MEM (Glasgow medium) and Vero cells in Medium 199 (Gibco Biocult Ltd). All media were buffered with 14mM Hepes and 15·6mM sodium bicarbonate and they contained 10% foetal calf serum for growing the cultures before infection.

Serum-free medium was used in infected cell cultures of all three cell lines; the serum was replaced by 0·2% yeast extract and 0·29% tryptose phosphate broth.

Fresh lung tissue from Hh1 which showed a considerable amount of consolidation was cut into small pieces almost 1 mm in size; they were washed three times in PBS containing 50 μg/ml gentamicin and 50 μg/ml fungizone. They were then distributed in several 25ml flasks (NUNC), cultured in BHK 21 medium (Glasgow minimal essential medium (MEM; Gibco Biocult) with 10% foetal calf serum, 14mM Hepes and 15·6mM sodium bicarbonate at 37° and left to adhere to the plastic.

Immunology.—The following diagnostic antisera were used: Canine distemper (horse, Wellcome Laboratories), measles, parainfluenza 1 (HA_2 and Sendai), 2 (Greer), 3 (HA_2) (all guinea pig, Flow Laboratories), rinderpest and Peste des Petits Ruminants virus (PPRV) (ox and goat sera, kindly supplied by ARC Laboratories, Pirbright), mumps and SV5 (rabbit, Oxford Public Health Laboratories); vesicular stomatitis strains Indiana and New Jersey 144 (rabbit, ARC Laboratories, Pirbright).

Serum samples from various hedgehogs were also used.

Electron microscopy.—Partially purified and concentrated materials separated from faecal suspensions and tissue cultures were fixed with 2% glutaraldehyde and observed with a JEOL transmission electron microscope. Nucleocapsids were examined after disruption of the virions with sodium dodecyl sulphate before fixation and were stained in the same manner as the virions.

Some sections of virus-infected cells were prepared and photographed at Bristol University.

Immunofluorescence. — Immunofluorescence was studied by the indirect or sandwich method using commercially obtained F.I.T.C.-labelled sera and a Zeiss Photomicroscope with a mercury vapour lamp and dark-ground optics.

Samples were fixed in cold acetone and air-dried, or freeze-dried. Smears from the affected hedgehog organs were also examined.

Neutralization tests. — Neutralization tests (NT) were done in flat-bottomed microplates (96 wells, Linbro, Flow.) with an approximate capacity of 0·35 ml per well. 0·1 ml of antigen and 0·1 ml of antisera were allowed to react for 1–2 h at 37°, and 3×10^4 cells in 0·1 ml were then added to each well. Neutralization tests were also done in confluent cell cultures grown in 25 ml plastic bottles (Falcon). In order to study the differential cytopathic effect (CPE) induced by a viral suspension in the presence of various antisera, enough virus to induce a rapid CPE was mixed with different concentrations of serum samples and then used to infect the cells. The virus/sera mixture was incubated for 1 h before inoculation.

Haemagglutination and haemolysis.—Haemagglutination (HA) was measured at 4° against 1% guinea pig red cells in PBS containing 0·005% gelatine and 2·14% glucose, in microplates. The same plates were then incubated at 37° to determine the haemolytic activity.

Ether sensitivity.—Isolates were tested for sensitivity to ether by shaking virus suspensions with an equal volume of ether at intervals for 10 min at room temperature after the evaporation of the ether.

RESULTS

Symptoms

A hedgehog found in a public park in midsummer initially attracted attention because of the unusual time of its activity (07.30 hours) and by its unusual movements. It repeatedly ran round in circles, invariably in a clockwise direction, with occasional pauses when it raised its head and sniffed the air. It walked with a stilted gait high on its feet and occasionally fell over when describing a tight circle. Released in a private garden, it collided with any object in its path but in a very short time seemed to have learnt the layout of the garden and showed a remarkable ability to avoid obstacles. When examined closely the hedgehog made no attempt to curl up or make any other defensive or aggressive movements.

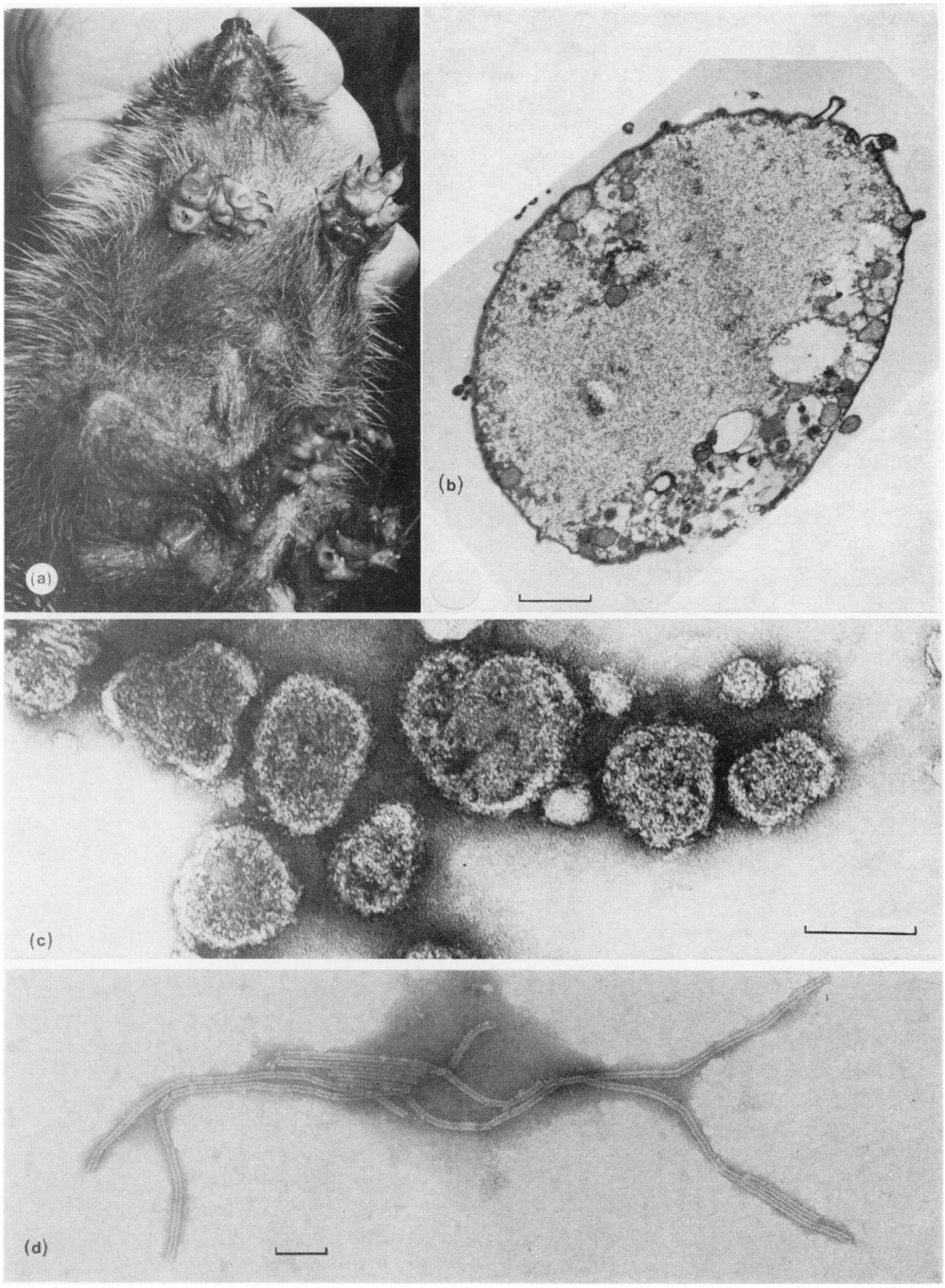

FIG.—(a) Ulcerative lesions on the feet of a hedgehog, Hh1, at time of capture. (b) Section through L929 cell infected with Hh1 isolate, showing paramyxo virus particles near cell surface and also budding from it. Scale bar = 1 μm. (c) Virus particles purified from faecal suspension of Hh1. Scale bar = 100 nm. (d) Nucleocapsids from the isolated virus. Scale bar = 100 nm.

The animal was a male weighing 750 g and at first sight, although small, appeared to be in relatively good condition and not particularly infected by ectoparasites. Its right eye was badly damaged, looking like a white ball, and later upon dissection it was found to be encased in fibrous tissue; its left eye was intact but had a glazed and cloudy appearance. The animal was obviously blind and did not react to movement close to it, provided no small air current was induced by the movement. Nor did it react to light flashing directly into its eye. It had sores around its mouth and on its face and a slight discharge was noticeable from both eyes and nose. The soles of its feet were ulcerated and swollen, with considerable thickening of the pads (hyperkeratosis). Its ankles and joints were also swollen and distorted (Fig. 1a).

Hh1 was kept in captivity; it escaped on one occasion but was recaptured after 2 weeks. It ate voraciously, maintaining its body weight for 2 months but then started to lose weight and rapidly became emaciated. It lost 37% of its body weight during its final 2 weeks. Its excreta were always loose and liquid during captivity. When *in extremis*, Hh1 was destroyed under anaesthesia.

Virus isolation in cell culture

Faecal samples of Hh1.—Samples of the first faecal suspension prepared from Hh1 soon after capture were inoculated on to cultures of all 3 cell lines. In all cases, a cytopathic effect was observed after a few days of incubation. The type of CPE seen in the different lines, however, showed considerable variation and further variation also became apparent within each cell line during successive passages. In early passages, in all cell lines, the most characteristic effect was the production of small rounded cells, whether attached to the monolayer or floating in the medium. In the 3 lines, the CPE, which was focal in distribution, included the development of rounded cells, singly or in grape-like bunches. Some cells were aligned like beads in a rosary. Swollen and translucent cells, spindle-shaped cells and cells with stellate morphology were frequent and sometimes appeared to radiate from a pile-up of rounded cells. Giant cells and vacuolated cells were also common. Plaques appeared in monolayers when piled-up cells detached. Sometimes holes enlarged and coalesced with others, while smaller gaps disappeared as dividing cells entered them and restored the monolayer. CPE rarely destroyed all the cells and remaining cells could be recultured. The characteristics of the CPE and the variations encountered were similar to those described for paramyxo viruses.

Later cell passages of the isolate were continued in L929 cells. CPE became less obvious and did not proceed to total destruction of the monolayer, which tended to reform and survive for considerably longer than in earlier passages. Since previous work had indicated that foetal calf serum might exert an inhibitory effect on viral CPE, some passages were made without it. The amount of infectious virus appeared to increase. Since the CPE seen did not often spread to kill all the cells in a culture and was frequently transient, titrations of the amount of infectious virus present in a given suspension was not very precise. Nevertheless, assuming that clear morphological changes represented a virus-induced CPE, some estimates were made. In serum-free medium the titre was of the order of 10^5 infectious units per ml while with serum the titre was about 10^{-1}. It was estimated from the number of particles seen by electron microscopy that the ratio of non-infective (defective) to infective particles was of the order of 10^6 in media containing foetal calf serum. The infectivity of virus suspensions in L929 cells was increased by passages in serum-free medium at terminal dilutions of 10^{-5} and 10^{-6}. Further increases were obtained when passages were made at 1- or 2-day intervals, as soon as the first CPE, in the form of multinucleate and giant cells, was observed. In this way, infective virus was demonstrated in the culture medium in dilutions up to 10^{-7} and the ratio of non-

infective to infective virus was reduced to between 10^2 and 10^3. Infectivity of the isolate was totally destroyed by treatment with ether.

Isolation from lungs of Hh1.—Isolation was also attempted from cultured explants of lung tissue from Hh1. Cells grew out from the explants in approximately 7 days and samples were taken from the supernatant culture medium over 2 or 3 weeks. Again passed into L929 and Vero cells or BHK cells CPE was seen in 24 h in the form of typical spindle-shaped and stellate cells. Unmistakable CPE was observed at 10^{-1} dilution as well as at 10^{-5} and 10^{-6}, but by the third day CPE was more advanced at 10^{-6} dilution than at 10^{-1} and 10^{-5}; cultures inoculated with various dilutions did not reach the same degree of CPE until the 8th day. The kind of CPE was similar to that seen in passages from faecal suspensions.

Electron microscopy

Virus particles were observed in clarified partially purified faecal suspensions of Hh1 (Fig. 1c). The virus was very pleomorphic and ranged in size from 100 to 300 nm. Most particles had external spikes, though some appeared to be naked and smooth-coated. The nucleocapsids varied in length, the largest seen being 1–2 μm long. The outside diameter of this component was 18 nm and the internal core was 5 nm (Fig. 1d). In sections of infected tissue cultures, virus particles of typical paramyxovirus morphology were seen budding from cell surfaces (Fig. 1b). None were seen in preparations of uninfected control cells.

Purified supernatant fluids from the original lung cultures contained virus particles similar to those seen in faecal material.

Production of haemagglutinin and haemolysin

Haemagglutinin was found in the initial virus passages in L929 cells after the original isolation from faecal material of Hh1 but tended to disappear in subsequent passages. It was clear but not very strong and the titres obtained were 1/8–1/32. The virus did not appear to elute from the red cells and when the plates were placed at 37° clear haemolysis was seen. The haemolytic activity was 1 or 2 units above that of haemagglutinin. The haemagglutinin titre was not affected by treatment with Tween/ether (Waterson, Rotl and Ruckle Enders, 1963; Norby, 1962).

Haemadsorption was also demonstrated in infected L929 and Vero cells, again using guinea pig red cells, even in later passages from which haemagglutinating and haemolytic activity had disappeared.

Neutralization tests

In tests in microwells with 18 different antisera including paramyxoviruses 1, 2 and 3, mumps, LCM, VS, SV5 and 4 members of the morbilli group, only the latter gave any neutralization. CPE developed in the presence of all the other antisera.

Antiserum against PPRV and, to a lesser extent, measles and canine distemper virus protected cell cultures against the CPE induced by virus isolated from both lung cultures and faecal samples of Hh1. No protection was obtained with antiserum against rinderpest. Some morphological changes were observed, however, even in those cultures which showed obvious protection but they were clearly different from the pattern of CPE seen in unprotected wells. In neutralization tests done in Falcon flasks of L929 cells infected with an inoculum which induced an extensive CPE in 72 h (4th passage of faecal material in L929 cells) and doubling dilutions of various antisera, the highest dilution of antiserum shown to protect was as follows: PPRV 1/320, measles 1/80, canine distemper 1/20 and rinderpest $< 1/10$.

Several attempts were made to neutralize the virus in microtrays. As there was some quantitative variability, the results are shown as the geometric mean.

Measles	1/160
Canine distemper	1/380
PPRV	1/538
Rinderpest	⩽10

In spite of the numerical difference the morbilli group of virus antisera was the only one to give clear signs of protection.

Immunofluorescence

Immunofluorescence was used to test for virus antigens in 3 cell lines inoculated with the early passages of Hh1 isolates and fixed 18–24 h after infection. It was also used to test smears of Hh1 organs and finally in the Auerbach plexus dissected from the lower ileum attached to the longitudinal layer of smooth muscle. As in the neutralization tests positive reactions occurred in antisera to viruses of the morbilli group. Occasional reactions occurred to parainfluenza 1 and to mumps virus.

These findings are considered qualitatively and are complementary to those from NT. The results are dependent upon a variety of factors such as the origin of the virus in test, the origin and history of the testing virus used for antisera production and the animals used for antisera production. PPRV antiserum, for instance, reacted strongly when the serum was made in goat (the original host of the virus) and to a much lesser degree when made in ox. There were also differences when the isolates were tested in different cell lines (Table). Measles antiserum reacted poorly to immunofluorescence (just as rinderpest reacted feebly in NT). Both these antisera reacted also, but weakly, in the dissected Auerbach plexus and of the 4 antisera thus tested canine distemper antiserum produced the strongest reaction.

TABLE.—*Immunofluorescence tissue culture 18 h after infection with Hh1 faecal suspension*

(a)

Serum	Cell types: L929	BHK	VERO
1. Measles (Guinea pig)	−	−	+ +
2. Canine distemper (Horse)	+ +	−	+
3. Rinderpest (Ox)	+ + +	+	+ +
4. P.P.R.V. (Goat)	+ +	+ +	−
5. Parainfluenza 1 (Guinea pig)	−	−	−

Immunofluorescent reactions of the Hh1 isolate against various morbilli group antisera in three cell lines.

(b)

Serum	Smears of Hh1: Intestine mucosa	Spleen	Salivary gland	Kidney
1. Measles	−	+ +	+	−
2. Canine distemper	+ +	+ + +	+ + +	+ +
3. Rinderpest	+ +	+ +	+	+
4. PPRV	−	+ +	+ +	+ +

Immunofluorescent reactions of Hh1 smears against the same antisera.
+ = Traces.
+ + + = Strong fluorescence.

Histopathology

Brain.—Focal lesions were observed in both grey and white matter. In some areas the lesions appeared as spongiform degeneration while in others degeneration of the neurones was observed. There was extensive hyperchromatism and degeneration of Purkinje cells in the cerebellum and some involvement of the granular layer. Very rarely, small haemorrhages were seen, but not cellular infiltration nor perivascular cuffing. Cytoplasmic inclusion bodies were seen only occasionally. There was some gliosis in areas of degeneration and demyelinization. The lesions correspond closely to the neuropathology of canine distemper as described by Innes and Sanders (1962).

Lung.—The lung showed consolidated bronchopneumonia and interstitial pneumonia but although morbilli group viruses were suspected to be the causative agent, no Warthin (giant multinucleate) cells were found.

Spleen.—Sections of the spleen showed large numbers of multinuclear giant cells: they were not similar to the Warthin cells of measles infection and were difficult to identify. Some stained lightly and tended to show eosinophilic cytoplasm, but similar cells have been described in the spleen of normal hedgehogs (Carlier, 1893). Smears of spleen nevertheless showed immuno-

fluorescence in these giant cells against viruses of the morbilli group. The strongest reaction was to canine distemper antiserum.

The pancreas, liver, kidneys and salivary glands showed no lesions.

Foot.—Longitudinal sections of the ball of the foot close to the ulcerative lesions showed a degree of hyperkeratosis. Some cellular degeneration was found in the germinal layer of the epidermis and dermal papilla. Nuclear inclusion bodies were seen close to the germinal layer. Occasional large eosinophilic cells were seen in the connective tissue of the dermis.

DISCUSSION

The symptoms of disease in Hh1 suggested that the causative agent might be either foot-and-mouth-disease virus (FMD) or a virus closely related to canine distemper virus. Although hedgehogs are known to be susceptible to FMD, the ulcerated lesions on the soles of the feet of this individual were not vesicular and were restricted to the ball of the foot, unlike the typical FMD condition described by McLaughlan and Henderson (1947). No FMD particles were found during EM inspection. BHK cell cultures were not destroyed by the virus and the hedgehog did not succumb to the disease as rapidly as would be expected with FMD in this species. The virus isolated was shown to be sensitive to ether and proved to be a member of the morbilli group of the paramyxoviruses, of which canine distemper virus is also a member.

Striking similarities were apparent between the pathological changes found in the hedgehog and those in dogs infected with canine distemper virus: the brain lesions were similar, a marked preference being shown for the Purkinje cells of the cerebellum; the foot lesions showed hyperkeratosis, with cell degeneration in the germinal layer of the epidermis and hair follicles; there were lung lesions and opacities in the cornea.

It may be argued that since paramyxoviruses have also been recovered from apparently healthy hedgehogs and we have not attempted to fulfil Koch's postulate, the isolation of the virus from the diseased animal did not prove that it had caused the symptoms observed. However, no alternative candidate was isolated and the physical symptoms and pathological history of the hedgehog were consistent with the presumption that the disease was caused by a virus of the morbilli group. The results of the immunological techniques used indicated that it was clearly a member of the group but did not show a closer relationship with one member rather than another. This may reflect the antigenic relationships that have been demonstrated between all four members of the group (Gibbs *et al.*, 1979) and may also be a consequence of the heterogeneity of the original wild-type virus population. Such heterogeneity is a common characteristic of the morbilli viruses. For instance, measles virus can be highly heterogeneous with regard to a number of characteristics such as temperature sensitivity and pathogenicity (Ju *et al.*, 1980) and clones of the L16 measles vaccine varied in their neurovirulence from minimal to extreme when inoculated intracerebrally into Macaque monkeys. Two out of 5 seropositive monkeys developed neuronal destruction (Sharova *et al.*, 1979).

Viruses of the paramyxo group are notoriously troublesome to isolate and maintain. During work *in vitro* variability plagues the investigator. Thus, sometimes a virus appears to be lost during passage in tissue culture but then reappears, perhaps reflecting variations in the numbers of defective particles. Again, CPE may change under the influence of antibody to a related virus. In order to control any selection that might be caused simply by culture in one particular cell line, three different cell lines were used with the hedgehog isolate and considerable variations were indeed observed between the lines in response to virus growth in the presence of the series of the antisera tested. The types of CPE observed cor-

responded to those described for paramyxoviruses but each cell type exhibited differences in CPE. When infected cells were incubated with dilutions of antisera to the 4 related morbilli viruses, there was a considerable amount of protection which varied with cell type; the type of CPE also varied with the antiserum and its dilution.

Considerable interest has been shown in the possible connection between multiple sclerosis and canine distemper following the increased incidence of cases of MS in the wake of an epidemic of canine distemper (Kurtze and Hyllested, 1975). Interesting observations on the consequences of transfer of virus between man and dog were made by Tijl *et al.* (1971) and Notermans *et al.* (1973), who inoculated dogs intracerebrally with material from patients with subacute sclerosing panencephalitis and found that some of the dogs developed signs of hard pad. A number of measles viruses with different characteristics have been isolated from patients with SSPE (Fraser and Martin, 1978) but the induction of lesions in the soles of the feet and in the cornea have previously been regarded as characteristic of canine distemper virus.

It appears that paramyxo viruses of the morbilli group are commonly present in hedgehogs and in populations of small rodents (Vizoso, 1968; Vizoso and Thomas, in preparation). These viruses may survive in wild communities and the carriers will form a reservoir; if the viruses spread across species barriers, as is likely, to pass from wild to domestic animals such as the dog, they may subsequently give rise to infections in man.

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